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Detection of Bacterial Endotoxins by Limulus Amoebocyte Lysate (LAL) Test- A Review

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Sneha. V. Kiran*, Shivalinge Gowda K P

Department of Pharmacology, Faculty of Pharmaceutical Sciences, PES University (HN Campus), Bengaluru, India.-560050

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ABSTRACT

Limulus amebocyte lysate (LAL) test is an aqueous extract of amebocytes from the horseshoe crab- *Limulus polyphemus.* According to the U.S. Food and Drug Administration, the LAL test can be substituted for the Pyrogen test conducted for the end-product testing of injectable drugs including biological products and medical devices. The quantification of endotoxins in the raw materials including water used for production and examining the endotoxin levels. The LAL test is substituted for the rabbit pyrogen test when there is a defined endotoxin limit in the drug product. The LAL reagent in the presence of an endotoxin becomes turbid and forms a gel-clot, the formation of turbidity depends upon the concentration of the endotoxin in the product it is inversely proportional. LAL is the preferred method of testing because it agrees with the European Pharmacopoeia Commission in favor of different testing methods to that of traditional animal-related tests.

INTRODUCTION:

RATIONALE: There are several different methods available for testing the presence of endotoxins, such as the in vivo rabbit pyrogen test and various in vitro alternatives one such method is the Limulus Amebocyte Lysate (LAL) test.

Endotoxins:

Endotoxins are components of the outer membrane of Gram-negative bacteria. The gram-negative microorganisms comprise of the external stronger wall which is comprised of peptidoglycan (otherwise called murein). Murein is a polymer comprising of sugars and amino acids that shapes work like layer outside the plasma film of microbes, framing the cell wall. In gram-negative microscopic organisms' peptidoglycan is a slight film beneath the peripheral layer it is otherwise called an envelope, known as lipopolysaccharide (LPS) which is the furthest slim covering of the peptidoglycan. LPS is pyrogenic and bacterial endotoxin is equivalent to lipopolysaccharide.[1] Endotoxins are lipopolysaccharides. LPS is composed of three regions. a) The O- specific polysaccharide region is a repetitive glycan polymer. The O-antigen containing 100 or more oligosaccharide units contains 3 to 4 monosaccharides. It is heterogeneous. b) The core polysaccharide component besides the Lipid A substructure is constituted of membrane-associated with an enzymatic activity that catalyzes the addition of monosaccharides to the core polysaccharide. c) Lipid A is a three-dimensional structure, having a long fatty acid side chains and is highly resistant, which can withstand high pressure and extreme heat.[2]

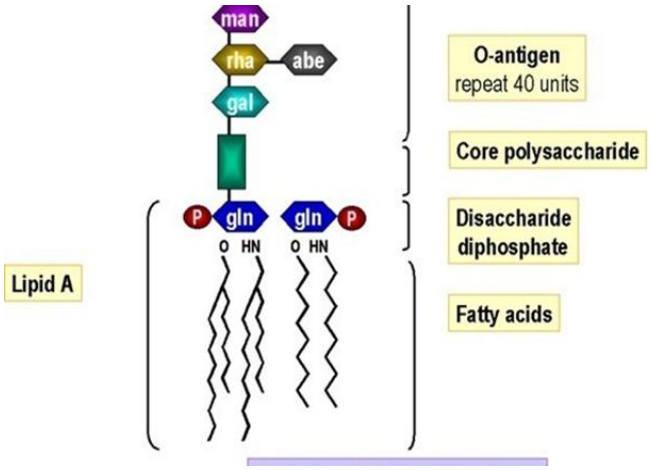

Figure No. 1: Structure of Endotoxin[3]

LAL TEST:

Bacterial endotoxin test (BET) is the strategy for the identification and quantitation of pyrogens or endotoxins. Wager measures and tests are suggested in all the pharmacopeias around the globe. The essential or the most utilized strategy is the LAL test (Limulus Amoebocyte Lysate Test) i.e., a reagent secured from the North American Horseshoe Crab: *Limulus polyphemus*. Thus, LAL is a fluid concentrate acquired after lysis of platelets (amoebocytes) from specific types of horseshoe crab.[4]

PRINCIPLE INVOLVED IN LAL TEST:

The LAL or Limulus test is utilized for the affirmation of bacterial endotoxins in a wide combination of tests in both research labs and organizations Endotoxin catalyzes the actuation of proenzyme in the LA lysate. The underlying pace of enactment is dictated by the convergence of present endotoxin. The initiated catalyst [coagulase] hydrolyzes an explicit bond inside a thickening protein [coagulogen] likewise present in LAL. Turbidity precipitation and gel structures can happen in the wake of blending bacterial endotoxin in with LA lysate. The LAL strategy utilizes lysate and that the lysate identifies endotoxin delivered from the lysis of Gram-negative microscopic organisms.

This identification depends on the regular biochemical thickening instrument which happens inside the horseshoe crab as a feature of its common guard against microorganism contamination. (The response is specific). The response of the horseshoe crab to endotoxin (the development of coagulation). Here, when endotoxin comes into contact with LAL it starts a progression of enzymatic responses that bring about the enactment of a pathway to the creation of in any event three serine protease zymogens (Factor C, Factor B, and proclotting compound). This pathway modifies amoebocyte coagulogen (an invertebrate fibrinogen-like clottable protein) to frame coagulin gel. A few reagents don't depend on the assortment of blood from the horseshoe crab; however, they are intended to work likewise. These reagents are recombinant renditions of Factor C, the enactment of which is the initial phase in the LAL response course.

Serine proteases are catalysts that separate peptide securities in proteins, in which serine fills in as the nucleophilic amino corrosive at the dynamic site. They are found in people just as in the horseshoe crab (and to be sure in all warm-blooded creatures). These proteases are liable for co-organizing different physiological capacities, including absorption, invulnerable

reaction, blood coagulation, and propagation. It is the blood coagulation response that is so comparative in people and the horseshoe crab. The LAL reagent utilized for the identification of bacterial endotoxins by the gel-cluster technique accompanies a distinguished affectability or name guarantee (lambda or λ), for instance, 0.03 endotoxin units (EU) per ml. On the off chance, that the test contains 0.03 or a greater amount of the reagent the example frames a gel or clusters. The unit of estimation for the LAL test is the Endotoxin Unit (EU), communicated as EU per mL or mg. These are a proportion of the movement of the endotoxin. Endotoxins contrast in their organic movement or strength; the pyrogenicity or LAL reactivity of one endotoxin planning might be altogether different from that of one more of similar weight. On the other hand, two endotoxin particles might be of various sizes and various loads however may have a similar reactivity in a LAL test. The strength of an endotoxin decided with one LAL reagent parcel may contrast from that decision with another part. Communicating endotoxin focuses on EU's maintains a strategic distance from the issues of various potencies of various endotoxins and permits microbiologists to look at aftereffects of changed LAL tests acted in various labs.

A few principles make reference to estimation for bacterial endotoxins as a worldwide unit (IU) given the RSE (reference standard endotoxin) which is fabricated from a unique strain of Escherichia coli microscopic organisms. Two principle worries in utilizing the LAL test is the nearness of meddling variables, for example, restraint or improvement of endotoxin and the over-weakening of tests, with the goal that a bogus negative is acquired, thinking little of the amount of endotoxin present in the example. Tests are furthermore run against a standard arrangement of anticipated endotoxin focuses and negative controls. Negative controls comprise of tests of LAL reagent water containing no noticeable endotoxin to which LAL reagent is included. Their motivation is to guarantee that the test framework doesn't give a sign without endotoxin and to confirm that the reagents are not sullied.[1,5]

CLASSIFICATION:[5]

Official pharmacopoeial method Bacterial endotoxins - LAL test: -

- Gel-clot method: limit test
- Turbidimetric method
- Chromogenic method

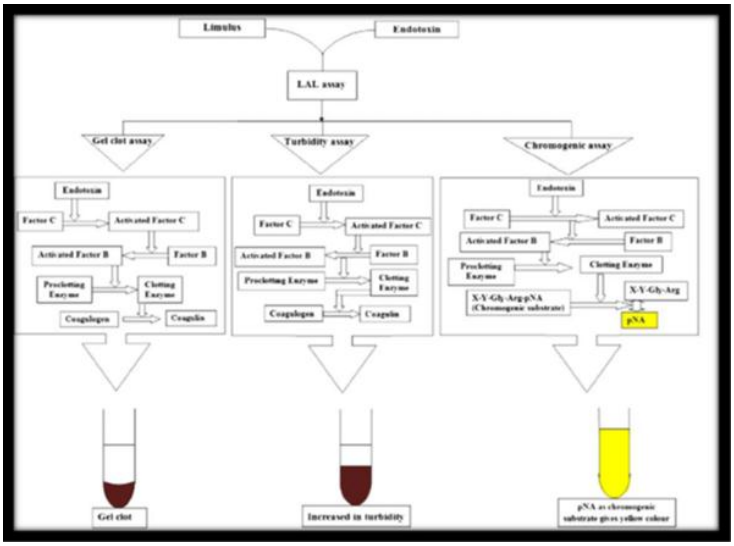

Figure No. 2: Flow chart of methods of toxin detection by LAL Test.[6]

Gel clot method:

The gel- clot method is a simple and convenient assay. It is a semi-quantitative method. It is the strategy for distinguishing endotoxins dependent on the wrapping of lysate within the sight of endotoxins. (Given the arrangement of gel). Equal parts of LAL are incorporated with endotoxin sample dilution, gelation can be observed. The concentration of endotoxins required to cause the lysate to cluster under standard condition is the marked lysate sensitivity. The concentration of endotoxin is calculated by multiplying the reciprocal of the highest dilution factor of sample.[5]

Kinetic Turbidimetric Method:

Turbidity leads to the formation of gel clot, hence a turbidimetric test is considered as the continuation of gel- clot assay. Formation of turbidity occurs in the LAL reagent, containing sufficient coagulation when cleaved by the clotting enzyme but inadequate to form a clot. The method establishes that the increase in endotoxin concentration causes a proportional increase in turbidity as a result of precipitation of coagulation in the lysate. It is a quantitative method employed for measuring endotoxin over an array of concentrations.[7]

Chromogenic substrate assay:

In the chromogenic method, the coagulogen is relatively replaced by a chromogenic substrate, a short synthetic peptide consisting of a sequence of amino acids on the verge of interaction with the clotting enzyme, resulting in the binding of the chromophore(para-nitroanilide) to the peptide end. The endotoxin- activated pro clotting enzyme is a definite advantage of the chromogenic method, here the para- nitroaniline is released in accordance to the increasing concentration of endotoxin when carboxyterminal glycine- arginine residue sequence gets conjugated with a chromogenic substance.[7]

PROCEDURE:

Gel clot method:

A standard solution having at least four concentrations equal to 2λ, λ, 0.5λ, and 0.25λ by diluting the Standard Endotoxin Stock Solution with water BET (bacterial endotoxin test). Mix a volume of the lysate with an equivalent volume of one of the standard solutions, (for example, 0.1 ml aliquots) in each tube. At the point when single test vials or ampoules, containing lyophilized lysate are utilized, add solutions of the standard into the vial or ampoule. Incubate the response mixture for a steady period as per directions of the lysate producer (ordinarily at 37±1°C for 60 ± 2 minutes), without vibration. Test the integrity of the gel for tests did in tubes, take each tube straightforwardly from the incubator, and reverse it through around 180 degrees in a single smooth movement. If a firm gel has shaped that remains in place upon reversal, record the outcome as positive. An outcome is negative if the gel isn't intact. The test is viewed as valid when the lowest concentration of the standard solutions shows a negative outcome in all cloned tests. The endpoint is the lowest concentration in the arrangement of declined concentrations of standard endotoxin that coagulates the lysate.[8] The geometric mean endpoint concentration is calculated by taking the mean of the logarithms of the endpoint concentrations of the four dilute solutions; take the antilogarithm of this value, as indicated in the following formula:

Geometric Mean Endpoint Concentration = antilog ∑ e/ f

∑ e = the sum of the log endpoint concentrations of the dilution series used

f = the number of replicate test tubes.

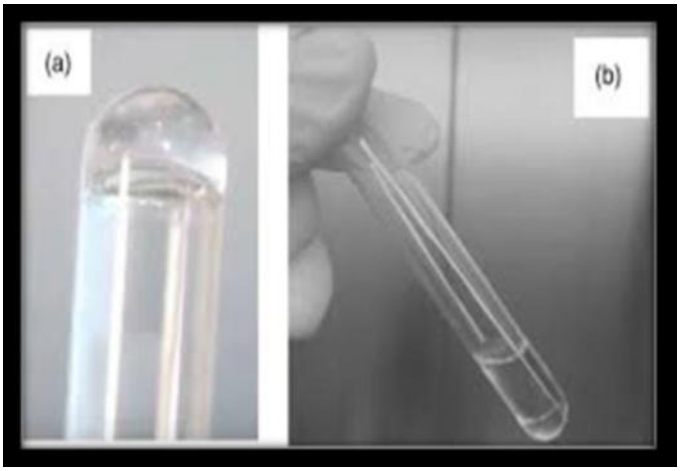

Figure No. 3: (a) Positive test –The solidification of gel and (b) Negative test – Gel is not solidified.[6]

Turbidimetric technique:

In the turbidimetric end-point method, the turbidity is measured at a determined period, which leads to errors and a disadvantage is that the reading cannot be taken simply and the entire process will need to be repeated if the reading is not taken at the indicated time, gelation continues. Hence for these reasons this method is not preferred. In the kinetic-turbidimetric method, it's a low limit of detection and wide range of endotoxin concentrations where the measurements take place can construct normal curves in a concentration range of up to 100 endotoxin units per milliliter of the solution.

PROCEDURE:

The procedure is a photometric test estimating an increase in reactant turbidity. Based on the specific measure guideline utilized, this procedure might be delegated either an endpoint-turbidimetric assay or a dynamic turbidimetric test. The endpoint-turbidimetric measure depends on the quantitative connection between the grouping of endotoxins and the turbidity (absorbance or transmission) of the reaction mixture toward the end of the incubation period. The kinetic turbidimetric assay is a technique to gauge either the time (beginning time) expected to arrive at a pre-determined absorbance or transmission of the reaction mixture or the rate of turbidity development. The test is done at the incubation temperature recommended by the lysate producer (which is typically 37 ± 1 °C).[8]

Chromogenic technique:

The test can be conducted by two methods: The kinetic chromogenic method involves measuring of color at different intervals of time after adding the LAL reagent containing the chromogenic reagent, to an endotoxin containing solution. The advantage of this method is the measurement of many samples in a short duration. The chromogenic endpoint method measures the appearance of color after the enzymatic reaction is done. The enzymatic reaction is over once the incubation period ends or the solution is acidified. One sample solution is measure at a time which is disadvantageous compared to the chromogenic kinetic method. It is a quantitative method of analysis and requires less incubation time compared to the gel-clot technique.

PROCEDURE:

The procedure is a test to gauge the chromophore discharged from a suitable chromogenic peptide by the response of endotoxins with lysate. Based on the particular assay utilized, this procedure might be classified as either an endpoint-chromogenic test or a dynamic chromogenic test. The endpoint-chromogenic method depends on the quantitative connection between the concentration of endotoxins and the end-point of chromophore toward the end of an incubating period. The kinetic chromogenic method is a way to quantify either the time (beginning time) expected to reach a predetermined absorbance of the reaction mixture and rate of color development. The test is completed at the incubation temperature suggested by the lysate producer (which is normally 37 ± 1 C).[8]

Advantages of the LAL test:[5]

- Less amount of testing sample is required for the LAL test.
- Multiple examples can be tested.
- Only one experienced laborer is adequate to carry on the tests.
- LAL test is progressively practical, despite having significant expenses.
- A lower level of endotoxins could be identified rather in the rabbit pyrogen test henceforth; this test is utilized as an option for endotoxin assurance.
- The LAL test is a standardized method of detecting endotoxins.

- Data for the last assessment of the test is obtained relatively fast.

Disadvantages of the LAL test:[5]

- Precise execution of the procedure can't be guaranteed.
- Slight disturbances and interferences in the equipment could impact the result of the test.

Applications:[9]

- LAL test has more application to the pharmaceutical business.
- The most common pyrogens are endotoxins in the pharmaceutical products; hence LAL test has been viewed as an alternative for the rabbit pyrogen testing by the European pharmacopoeial board of trustees.
- One significant explanation that has made LAL test a win in the pharmaceutical business is the cautious evasion by the LAL makers towards hurting live creatures during both creation and testing.
- It is essential to explain that the crabs, from which part of the hemolymph utilized for the LAL test was extricated, are returned alive to their common living space with no enduring issues after the extraction.

SUMMARY

Sterile pharmaceutical products screened by LAL test ensures that they are safe for human consumption, endotoxins are present in the bacterial structural component which are released when the bacterial cell is lysed. The endotoxins are toxic in nature and if administered causes a rise in body temperature deemed as pyrogenic response. LAL test is the most common approach for testing of endotoxin content in drugs, medical devices and implants. LAL test is an accurate and rapid test, the introduction of recombinant based LAL reagent has made it easier for testing of endotoxins compared to the original LAL assay. The test has been well established as an endotoxin test.

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Image *Author -1*	*SNEHA. V. KIRAN* *Post graduate student* *PES UNIVERSITY, (HN Campus) BSK I Stage, Bengaluru-560050*
Image *Author -2*	*SHIVALINGE GOWDA K P* *Professor and Chairperson,* *Department of Pharmacology* *PES UNIVERSITY, (HN Campus) BSK I Stage, Bengaluru-560050*