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REVIEW ARTICLE

ATTRIBUTES OF BACTERIAL ENDOTOXIN TEST (BET) AND ITS COMPARISON WITH RABBIT PYROGEN TEST.

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Abstract

Presence of pyrogen (mostly endotoxins) in therapeutics can cause harmful symptoms in patients, so it is mandatory to test the presence of pyrogens in different therapeutics including biologicals. Now-a-days the use of animals is not the preferred method for performance of quality control testing to detect the presence of pyrogens. As **Committee for the Purpose of Control and Supervision of Experiments on Animals** (CPCSEA) has introduced the 4R's i.e. Replacement, Reduction, Refinement and Rehabilitation. This has shifted the approach from *in-vivo* to *in-vitro* methods. Considering the 4R's, the Bacterial Endotoxin test (also known as Limulus Amebocyte Lysate Test) has been introduced as alternative method for Rabbit pyrogen test. The current article primarily focuses on comparison of Limulus Amebocyte Lysate Test (LAL test) and Rabbit pyrogen test and came to conclusion that, time taken for performing the Rabbit pyrogen test is approximately 6 hours apart from conditioning the rabbits for 1-3 days. Whereas, Limulus Amebocyte Lysate Test can be performed within 1-2 hours. Secondly, the sensitivity of LAL Test is more than Rabbit pyrogen test for endotoxin, Lipid A and plasma proteins, the range may vary. Lastly, various Pharmacopoeias like European Pharmacopoeia, British Pharmacopoeia, United States Pharmacopoeia and Indian Pharmacopoeia has already included the LAL Test as a replacement method in parenteral preparation and alternative method for Blood products.

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Introduction:-

The comparison between two techniques of pyrogen testing: Rabbit Pyrogen test and Bacterial Endotoxin test is carried considering its applicability, sensitivity, feasibility, and safety.

In 1956, when Fred Bang (MBL scientist) was studying the circulation of blood in horseshoe crabs, he noticed that one of the crabs from experimental group died due to result Vibrio bacterial infection. After analysis it was observed that the entire blood of the crab clotted into a firm semi-solid mass. Further investigation concluded that only gram-

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negative bacteria produce this strange reaction. It was noted that the reaction observed was a kind of endotoxin reaction that is similar to that observed in mammals, known as Schwartzman reaction (Schwartzman- is type of reaction that occurs if human tissue is exposed to endotoxin causes thrombosis). [29]

Background

Limulus Amebocyte Lysate (LAL) test received FDA approval in the mid of 1970's. FDA mentioned all the condition for testing finished product, blood products, disposable pharmaceuticals devices and intravenous fluids using LAL. It's almost more than past 30 years, Food and Drug Administration (FDA) had approved the use of LAL test for endotoxins parallel to the rabbit pyrogen test (FDA approval in 1940's). [12,16] USFDA in 1987 has established the guidelines for LAL test in terms of regulatory requirements with amendment in 1991. Similarly, United State Pharmacopoeia (USP), European Pharmacopoeia (EP), Japanese Pharmacopoeia (JP) and Germany has established the guidelines in USP 23, EP Supplement 1998, JP XIII, and 1993 respectively. [17] Indian Pharmacopoeia included monographs for replacement of pyrogen testing for human vaccines and biotech product was included in IP 2014. BET can be used as alternative method instead of pyrogen test for blood products, if validation and consistency is established. [28]

Bacterial Endotoxin

The cell walls of gram-negative bacteria constitute Bacterial Endotoxin comprising of Lipopolysaccharide (LPS). The molecular weight of the LPS molecules varies from 1000 to 25,000Da. It is pyrogenic in nature which are toxic to human if exposed via intravenous and intramuscular preparation. The toxicity of the endotoxin, when injected, results in rapid increase of body temperature often followed by severe and rapid shock, which leads to death before the diagnosis of the cause. [10,30]

The endotoxin toxicity is associated with the lipid component of bacteria i.e. Lipid A whereas, polysaccharides are responsible for immunogenicity. The cell wall antigen also commonly known as O-antigens are components of LPS of gram-negative bacteria. Endotoxin are thermostable (i.e. if heated for 30 minutes do not get destroyed or destabilize), but few authors have reported that these endotoxins can be neutralize with the help of superoxide, peroxide and hypochlorite. [14]

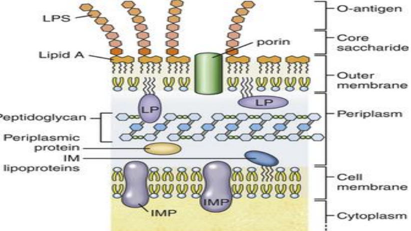

Fig 1: -Schematic diagram of gram-negative infection (Jane E. Sykes, Gram-negative bacterial infection, Chapter 36, veterian key).

1.2.1 Chemistry of Endotoxin

As mentioned before Endotoxin are comprised of Lipopolysaccharides (LPSs), they are macromolecules consisting of two covalently linked part: a hydrophilic polysaccharide which further subdivided into two parts – O-polysaccharide and core polysaccharide, and a hydrophobic lipid region i.e. the lipid A. [4]

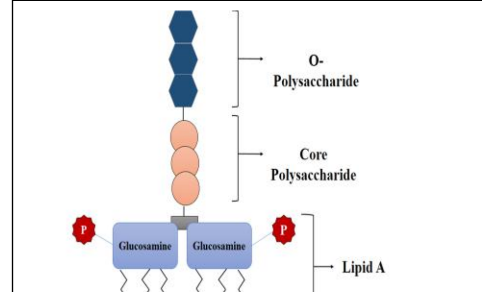

Fig 2:-Diagrammatic representation of Endotoxin and its part

These three components of LPS – Lipid A, Core polysaccharide, and O-polysaccharide are described below: [4, 9, 22]

Lipid A:
It is the lipid component containing the hydrophobic, membrane-anchoring region of LPS. It consists of phosphorylated N-acetylglucosamine (NAG) dimer attached with 6 or 7 saturated fatty acids (FA). Some of the FA are directly attached to NAG while some are esterified to the 3-hydroxy fatty acid. The biological activity of lipid A depends upon particular conformation that is determined by the glucosamine disaccharide, the PO_4 groups, the acyl chains, and also the KDO-containing inner core [14]. In salmonella species, lipid A consist of a biphosphorylated disaccharide of D-glucosamine in β-1, 6-linkage. The disaccharide part bears amide and ester linked residues of 3-hydroxymystristic acid, which perhaps get esterified themselves with lauric, myristic and palmitic acid.

Core Polysaccharide:
It is also known as R antigen. It is attached to the 6th position of one NAG dimer. It consists of short chain of sugar such as heptose and 3-deoxy-d-manno-2-octulosonic acid (KDO), are commonly present in core polysaccharide [14]. In salmonella species, core polysaccharide consists of oligosaccharide containing D-galactose, D-glucose, L-glycero-D-mannose-heptose, N-acetyl-D-glucosamine and KDO.

O-Polysaccharide:
It is also known as O-antigen, attached to the core polysaccharide. It is comprised of repeating oligosaccharide subunits made of 3-5 sugars. The length of the O-Polysaccharide is much longer than core polysaccharides with varying length ranging up to 40 repeat/unit [14]. It helps in characterizing structural diversity among various bacterial genera as well as among different serotypes of one and the same genus.

Lipid A part of the LPS is solely responsible for the endotoxic activity, while both the polysaccharides are deprived of toxic properties. All three components of LPS are immunogenic, which evoke the production of antibodies specific for structures in respective component of LPS.

Biological Properties Of Endotoxin
Endotoxin are the pyrogens (fever-inducing agents), and its pyrogenicity was first ever activity that had been recognized. Endotoxin are well-endowed with large spectrum of biological activities that can be studied in vivo and in vitro. Examples of endotoxic activities due to pyrogenicity includes leukopenia, leukocytosis, and induction of lethal shocks.

Despite the fact that endotoxin is named after their toxic properties, they also exhibit some of the beneficial activities, such as [4]:
1. Induce nonspecific resistance to various infectious agents and their toxic effect
2. Induce mitogenic stimulation and antibody synthesis and secretion in mouse B lymphocytes.

Hemolymph of Horseshoe Crab
There are four different species of horseshoe crabs, *Tachypypleus tridentatus, Tachyphleus gigas, Carcinoscorpiou rotundicauda* and *Limulus polyphemus*. Only *Limulus polyphemus* resides in east coast of North America while other three resides in Southeast Asian coast. *Limulus* belongs to class arthropod, which are more closely related to spider than crabs. Although the circulatory system of *Limulus* is much more open, all other arthropod species possess a semi-closed circulatory system [29]. *Limulus* are cold-blooded i.e. they can't raise the body temperature. The limulus hemolymph has two type of cells: granular hemocytes and cyanocytes depending upon the cell morphology there is only one type of hemocyte in adult intermolt animal's systemic circulation that is called granulocyte or amebocyte. The morphology is such that cell is oval, plate-shaped structure with 15-20µm in dimensions. The cells also contain plentiful dense granules, some are large (L) granules up to 1.5µm in diameter and other are small(S) granules. [3]

The L-granules comprises of at least 20 protein components along with four clotting factors and one antimicrobial factor (anti-LPS factor), whereas S-granule contain six major protein component and some other antimicrobial substances. [1,3,8,18,21,31] The blood of the horseshoe crab is blue in colour because it contains protein called Hemocyanin, function as oxygen-carrying pigment. The molecular cascade of blood coagulation in Limulus polyphemus consist of four serine protease zymogens (i.e. Factor C, Factor G, Factor B, and Proclotting factor) and

one clottable protein (coagulogen) [27]. The diagrammatic representation of proteolytic cascade in *L. polyphemus* is shown below:

Fig 3:-Illustration of proteolytic cascade of blood coagulation after invading of micro-organism, which activates serine protease zymogens leads to conversion of soluble coagulogen into insoluble coagulin. Hence, formation of semisolid mass take place in larger granules of horseshoe crab hemocyte.

Comparison Of LAL Test With Rabbit Pyrogen Test

Primarily, LAL test is an in-vitro test. Whereas, Rabbit pyrogen is in-vivo test. The LAL test is used to detect the bacterial endotoxins in drugs and biological product. LAL is aqueous extract of amebocytes from the horseshoe crab, *Limulus polyphemus*. Bacterial endotoxins or LPSs, which is outer membrane component of gram-negative bacteria, when encounters with LAL form a gel clot within 1 hour at normal physiological temperature i.e. 37 ºC. The tube used in BET should be depyrogenated before using and if possible, test should be performed in bioassay cabinet to avoid entry of contamination.

On the other hand, rabbit pyrogen test is carried out in 2 steps: Preliminary test and Main test. In preliminary test, rabbit is selected and conditioned for 1-3 days. Following injection of pyrogen free saline solution warmed to about 38.5 ºC and the temperature of animal is to be recorded using graduated thermometer at 30-minute interval for 3 hours after injection. Animals those showing temperature variance of 0.6 ºC should not be selected for main test. In main test, selected 3 groups for rabbit from preliminary test are used and temperature is to be recorded of each animal for 3 hours at 30 minutes' interval before injection and continuing for 3 hours after the injection. Initial temperature of each rabbit which is mean of two temperatures recorded for that rabbit at 30-minute time interval. Rabbit showing temperature variance > 0.2ºC between two successive readings should be excluded. Only those should be selected whose initial temperature do not vary by more than 1ºC. Each selected rabbit is injected with sample into marginal vein of the ear. The volume of injection is not less than 0.5ml/kg and not more than 10ml/kg of rabbit's body weight. Temperature is to be recorded during 3 hours at 30 minutes' intervals. Interpretation of test is done and the test passes if the sum of the raise temperature in 3 rabbit does not exceed 1.4 ºC and response of each rabbit is not more than 0.6 ºC. If response of any of the rabbit is 0.6 ºC or more than that or sum of response of 3 rabbit exceed 1.4 ºC then again test was repeated in additional 5 rabbits. If the sum of raise temperature in all 8 rabbit does not exceed 3.7 ºC or if not more than 3 of 8 rabbit show individual response of 0.6 ºC or more than it, then test was passes for that preparation. For various Biological products pyrogen test has been replaced by BET in monographs of Indian Pharmacopoeia (IP) 2018 (Table: 1) and BET is also used as an alternative method for pyrogen test for some Biological products in IP 2018 [28, 11] (Table: 2)

Table 1: -Biologicals Products for which pyrogen test has been replaced by BET in IP 2018

Human Vaccine	Biotech Product
• Hepatitis A (inactivated) and hepatitis B (rDNA) vaccine • Inactivated hepatitis A vaccine (adsorbed) • Inactivated influenza vaccine (split virion) • Inactivated influenza vaccines (surface antigen) • Inactivated influenza vaccines (whole virion) • Japanese encephalitis vaccine (human) • Poliomyelitis vaccine (inactivated) • Tetanus vaccine (adsorbed) • Typhoid polysaccharide vaccine • Yellow fever vaccine (live)	• Erythopoietin-concentrared solution • Erythropoietin for injection • Erythropoietin injection • Filgrastim-concentrated solution • Filgrastim injection • Insulin aspart • Insulin aspart injection • Insulin lispro • Insulin lispro injection • Biphasic insulin lispro injection • Biphasic insulin aspart injection • Isophane insulin injection • Interferon α2 concentrated solution • Interferon α2 injection • Interferon α2b injection

Table2: -Blood products for which BET is an alternative method for pyrogen test in IP 2018

Blood Products
• Human albumin • Human coagulation factor IX • Human coagulation factor VII • Human normal immunoglobulin • Human prothrombin complex • Human normal immunoglobulin for intravenous use • Antithrombin III concentrate • Plasma (pooled and treated for virus inactivation) • Blood grouping reagents (x7) • Blood grouping serums. • Rabies Immunoglobulin • Tetanus immunoglobulin • Whole Human Blood

Sensitivity of LAL test Compared to Rabbit Pyrogen

Various studies have mentioned that LAL test is more sensitive than the rabbit pyrogen test. One of the study compared LAL with rabbit pyrogen test (European Pharmacopoeia) on 50 samples of aqueous fluids, 22 samples of various medicinal preparations, 12 samples of biologicals, 11 samples of antibiotics with regard to various endotoxins, they concluded that LAL was as sensitive as rabbit pyrogen for endotoxin from K. pneumonia and at least 10 times as sensitive for other endotoxins tested. They also examined the contaminated glucose and sodium chloride infusion fluid that showed greater sensitivity in the LAL test. [6-7,13] Another study stated that LAL test is 3 to 300 times more sensitive to endotoxin than USP pyrogen test. They also tested a hypothesis that different preparations of LAL might not show specific reaction to endotoxin molecules, or different LAL preparation showing different results. Finally concluded that, the discrepancy observed might be due to differences in manufacturing process of LAL, causing alteration in LAL specificity to react with endotoxins [7,26] (Table: 3). Some specific endotoxins can be detected up to 0.03 ng/ml by LAL test. [26] The LAL test is more sensitive in testing plasma proteins, also it is tenfold more sensitive than rabbit pyrogen test for endotoxin and lipid A of gram-negative bacteria. LAL test is an additional method in detection for presence of lipopolysaccharides in drugs [24]

Table3: -Sensitivity of LAL preparation from different manufacturers with various endotoxins. [26]

LAL source	Endotoxin [a] (ng/ml)					
	E. coli	*S. typhosa*	*S. marcescens*	*S. flexneri*	*P. mirabilis*	*Pseudomonas spp.*
Single-test vial						
Microbiological Associates	0.3	0.4	0.5	0.5	700	30
Difco	0.3	0.1	0.3	0.3	200	20
Multitest vial						
Mallinckrodt	0.03	0.04	0.06		30	80
Sigma chemical	0.06	0.04	0.06	0.06	30	50
Difco	0.06	0.04	0.06		40	10
Associates of Cape cod	0.06	0.06	0.08	0.08	100	500

Advantages of BET Over Rabbit Pyrogen Test

Various studies concluded that the sensitivity of the BET is more than that of rabbit pyrogen test for parenteral preparation, pharmaceutical products, plasma proteins etc. apart from some exception. There are disadvantages that have been reported for rabbit pyrogen test that shows the superiority of BET over rabbit pyrogen test. Rabbit pyrogen test has drawback for testing large number of samples, since large stock of animals are needed as once there is positive response in the rabbit it blocks the further use of same animal for minimum of 3 weeks' period. The time taken for performing Rabbit pyrogen test is almost 6 hours, while LAL test is performed within 1-2 hours. Also, Rabbit Pyrogen test has low accuracy of reaction than LAL test due to elevation of temperature of animal body may result in need for repetition of test in a greater number of animals. [23]

Challenges of Replacing Rabbit Pyrogen Test To BET

Presently, Rabbit Pyrogen test is replaced by BET in parenteral preparations, human vaccines, biotech products, medical devices, etc. except in Blood products. BET is limited for testing of gram-negative bacterial endotoxin in the product, while Rabbit Pyrogen test can be used for detecting other pyrogens besides endotoxins. [23] Some limitation of BET which yielded discrepancy in results such as interference of mimicking substance that lead to false positive or false negative result, such as false negative LAL test in 20% chloramphenicol monosuccinates, glutathione and few lipids as they inhibit clot formation in 1:16 dilution. [15] Few studies reported that viral vaccines can also give LAL test negative even they have positive rabbit pyrogen test. [25] It also shows false positive test results for cellulose and many of herbal preparations [32]. Some studies also observed negative results for LAL with addition of endotoxin in presence of calcium ≥0.34 M and tetracycline HCL solution. [13] Therefore, test for interference is necessary before performing test for different batches. Overall, Limulus test or BET is beneficial alternative method for detection of pyrogen test.

Conclusion:-

Transition of BET as an alternate method to detect the pyrogen held to be beneficial in terms of time, effectivity and reduction of animal usage in experiment. The animal welfare in India is been promoting this alternative method with the objective that enhance animal well-being. Therefore, CPCSEA is aiming to implement 4R strategy- replacement, reduction, refinement and rehabilitation. 1) Replacement defines about avoiding or replacing use of animal 2) Reduction defines minimizing the number of animals 3) Refinement defines to minimize suffering, modernize experiment & improve animal welfare 4) Rehabilitation defines the care of animal post experimentation [2,5,28]. In latest version of Indian Pharmacopoeia (IP) 2018, Rabbit pyrogen test has been replaced by LAL test for various Biologicals including Human vaccines and Biotech products, while for various Blood Products LAL test is used as an additional method along with Rabbit Pyrogen test to establish LAL test as an alternative method to replace Rabbit Pyrogen test in Blood Products. Although, LAL test is more sensitive than Rabbit Pyrogen test, but it can lead to discrepancies in results due to presence of interfering compounds in the samples. Further investigation is required to overcome these discrepancies.

Future Prospect

BET has already established on parenteral preparations & to establish BET completely for all therapeutics requires further more analysis in specific products and after comparing the BET results with that of Rabbit Pyrogen Test can replace the Rabbit Pyrogen test to BET so as to detect the presence of any pyrogen for rest of the therapeutics i.e.

Biologicals, blood products etc. So, that it can be used as an alternative method to fulfil the requirements of CPCSEA 4Rs policy.

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