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Bacterial endotoxins

What are bacterial endotoxin?

An endotoxin is a lipopolysaccharide (LPS) found in the cell wall of gram-negative bacteria. It is a typical pyrogen, which induces various biological reactions when even a small amount of pg (10-12 g) or ng (10-9 g) enters the bloodstream. Due to its heat resistance and stability, complete inactivation of endotoxin is not possible with autoclaving. Dry heat sterilization for at least 30 minutes at a temperature of 250 °C or more is required complete inactiocaiton,. It exists in the environment (e.g. water, air) inhabited by gram-negative bacteria, and bacterial endotoxins (LPS) remain even after the bacteria die.The bacterial endotoxin test (BET) is one such quality control test. The BET using gel clot method is a 60-min test used determine the endotoxin content. The gel clot is technically the most simple and was the first BET approved by the Food and Drug Administration (FDA). Endotoxin is a subset of pyrogens that are strictly of gram-negative origin, a natural complex of lipopolysaccharides occurring in the outer layer of the bilayered gram-negative bacterial cell. From the circulating blood cells of Limulus polyphemus, called amebocytes, a clear lysate is obtained that forms an opaque gel in the

presence of extremely low concentrations of bacterial endotoxins.

Effects of endotoxin

Septic shock occurs during severe infections with Gram-negative organisms when bacteria or lipopolysaccharide enter the bloodstream. Endotoxin acts on neutrophils, platelets and complement to produce, both directly and through mast cell degranulation, vasoactive amines that cause hypotension.

Most times when bacterial endotoxin comes in contact with someone it causes sepsis leading to septic shock and then leading to DEATH

TEST FOR BACTERIAL ENDOTOXINS

The bacterial endotoxins test (BET) is a test to detect or quantify endotoxins from Gram-negative bacteria using amoebocyte lysate from the horseshoe crab (Limulus polyphemus or Tachypleus tridentatus). There are three methods for this test:

• Method A. The gel-clot technique, which is based on gel formation;

• Method B. The turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate;

• Method C. The chromogenic technique, based on the development of color after cleavage of a synthetic peptide-chromogen complex.

All glassware were depyrogenated and other heat stable materials in a hot air oven using a validated process. A commonly used minimum time and temperature is 30 minutes at 250°C. If employing plastic apparatus such as microplates and pipet tips for automatic pipetters, use apparatus shown to be free of detectable endotoxin and which does not interfere in the test.

REAGENTS AND TEST SOLUTIONS

Amoebocyte lysate

A lyophilized product obtained from the lysate of amebocytes (white blood cells) from the horseshoe crab (Limulus polyphemus or Tachypleus tridentatus).

Note: Amebocyte lysate reacts to some β-glucans in addition to endotoxins.

Amebocyte lysate preparations which do not react to glucans are available: they are prepared by removing the G factor reacting to glucans from amebocyte lysate or by inhibiting the G factor reacting system of amebocyte lysate and may be used for the endotoxin testing in the presence of glucans.

Lysate TS

Dissolve amebocyte lysate in water BET or in a buffer recommended by the lysate manufacturer, by gentle stirring. The reconstituted lysate,was stored refrigerated or frozen, according to the specifications of the manufacturer.

Water BET

Water for injections or water produced by other procedures that shows no reaction with the lysate employed, at the detection limit of the reagent.

Preparation of Standard Endotoxin Stock Solution

A Standard Endotoxin Stock Solution is prepared from an endotoxin reference standard that has been calibrated against the International Standard for endotoxins.

Endotoxin is expressed in International Units (IU) of endotoxin.

Note: One International Unit (IU) of endotoxin is equal to one Endotoxin Unit (EU).

Preparation of Standard Endotoxin Solution

After mixing the Standard Endotoxin Stock Solution vigorously, the appropriate serial dilutions of Standard Endotoxin Solution, were prepared using water BET.

Use dilutions as soon as possible to avoid loss of activity by adsorption.

Preparation of sample solutions

The sample solutions were prepared by dissolving or diluting drugs using water BET. Some substances or preparations may be more appropriately dissolved or diluted in other aqueous solutions. If necessary, adjust the pH of the solution to be examined (or

dilution thereof) so that the pH of the mixture of the lysate TS and sample solution falls within the pH range specified by the lysate manufacturer, usually 6.0 to 8.0. The pH may be adjusted by the use of acid, base, or suitable buffer as recommended by the lysate manufacturer. Acids and bases may be prepared from concentrates or solids with water BET in containers free of detectable endotoxin. Buffers must be validated to be free of detectable endotoxin and interfering factors.

DETERMINATION OF MAXIMUM VALID DILUTION

The Maximum Valid Dilution (MVD) is the maximum allowable dilution of a sample at which the endotoxin limit can be determined.

Determine the MVD from the following equation:

$$MVD = \frac{\text{Endotoxin Limit} \times \text{Concentration of sample solution}}{\lambda}$$

METHOD A: GEL-CLOT TECHNIQUE

The gel-clot technique is for detecting or quantifying endotoxins based on clotting of the lysate TS in the presence of endotoxin.

The minimum concentration of endotoxin required to cause the lysate to clot under standard conditions is the labeled sensitivity of the lysate TS. To ensure both the precision and validity of the test, the tests were performed for confirming the labeled lysate sensitivity and for interfering factors.

Test for confirmation of labeled lysate sensitivity

In four replicates the labeled sensitivity, λ, expressed in IU/ml of the lysate prior to use in the test. The test for confirmation of the lysate sensitivity is to be carried out when a new lot of lysate is used or when there is any change in the test conditions which may affect the outcome of the test.

The standard solutions were prepared having at least four concentrations equivalent to 2λ , λ, 0.5λ and 0.25λ by diluting the Standard Endotoxin Stock Solution with water BET.

A volume of the lysate TS was mixed with an equal volume of one of the standard solutions (such as 0.1 ml aliquots) in each tube. When single test vials or ampoules, containing lyophilized lysate are employed, add solutions of standards directly to the vial or ampoule. Incubate the reaction mixture for a constant period

according to directions of the lysate manufacturer (usually at 37±1°C for 60 ± 2 minutes), avoiding vibration. Test the integrity of the gel for tests carried out in tubes, take each tube in turn directly from the incubator and invert it through approximately 180 degrees in one smooth motion. If a firm gel has formed that remains in place upon inversion, record the result as positive. A result is negative if an intact gel is not formed.

The test is considered valid when the lowest concentration of the standard solutions shows a negative result in all replicate tests.

The endpoint is the lowest concentration in the series of decreasing concentrations of standard endotoxin that clots the lysate. Determine the geometric mean endpoint concentration by calculating the mean of the logarithms of the endpoint concentrations of the four dilution series, take the antilogarithm of this value, as indicated in the following formula:

Geometric Mean Endpoint Concentration = antilog

$$\frac{\sum e}{f}$$

$\sum e$ = the sum of the log endpoint concentrations of the dilution series used

f = the number of replicate test tubes

The geometric mean endpoint concentration is the measured sensitivity of the lysate (IU/ml). If this is not less than 0.5λ and not more than 2λ, the labeled sensitivity is confirmed and is used in tests performed with this lysate.

METHOD B. TURBIDIMETRIC TECHNIQUE

This technique is a photometric assay measuring increases in reactant turbidity. On the basis of the particular assay principle employed, this technique may be classified as either an endpoint-turbidimetric assay or a kinetic-turbidimetric assay.

The endpoint-turbidimetric assay is based on the quantitative relationship between the concentration of endotoxins and the turbidity (absorbance or transmission) of the reaction mixture at the end of an incubation period.

The kinetic-turbidimetric assay is a method to measure either the time (onset time) needed to reach a predetermined absorbance or transmission of the reaction mixture, or the rate of turbidity development.

The test is carried out at the incubation temperature recommended by the lysate manufacturer (which is usually 37 ± 1°C).

METHOD C. CHROMOGENIC TECHNIQUE

This technique is an assay to measure the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with lysate. this technique may be classified as either an endpoint -chromogenic assay or a kinetic-chromogenic assay.

The endpoint-chromogenic assay is based on the quantitative relationship between the concentration of endotoxins and the release of chromophore at the end of an incubation period.

The kinetic-chromogenic assay is a method to measure either the time (onset time) needed to reach a predetermined absorbance of the reaction mixture, or the rate of color development.

The test is carried out at the incubation temperature recommended by the lysate manufacturer (which is usually 37 ± 1°C).

Safety test

safety assessment, or toxicity testing, is the process of determining the degree to which a substance of interest negatively impacts the normal biological functions of an organism, given a certain exposure duration, route of exposure, and substance concentration. Toxicology testing is often conducted by researchers who follow established toxicology test protocol for a certain substance, mode of exposure, exposure environment, duration of exposure, or for a particular organism of interest, or for a particular developmental stage of interest. Toxicology testing is commonly conducted during preclinical development for a substance intended for human exposure. Stages of in vitro and in vivo research are conducted to determine safe exposure doses.

Safety tests are short duration single application test to evaluate over toxicity. The test are evaluated for gross evidence of toxicity such as survival and/or body weight loss. During the period of time the expected outcome of a toxicity free sample is weight gain, good health while the expected outcome of a toxicity filled sample is weight loss and death.

Safety test as done in the in vivo laboratory was carried out by administering drugs that are undergoing safety or toxicology

testing and administered to laboratory animals and these animals are then observed for a period of 7 days and carefully observed for loss of weight, I'll health or death.