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TAL - a source of bacterial endotoxin detector in liquid biological samples

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Abstract: Endotoxins (chemically known as Lipopolysaccharide) from gram-negative microorganisms initiates clot formation in blood when it is accidentally encountered by horseshoe crab blood stream. This property was extensively studied by various researchers as a result Limulus Amebocyte Lysate (LAL) test was established. The LAL tests in general, 3 to 300 times more sensitive than the United States Pharmacopeial (USP) rabbit pyrogen test method. It is apparent that major differences among the LAL preparations lie in the area of sensitivity. Differences, up to 100-fold, exist in the sensitivity of the various LAL preparations to the same endotoxin. Based on the above perspective, a portable Kit (Endo sensor) was developed to detect the presence of bacterial endotoxin in liquid biological samples using Tachypleus Amebocyte Lysate (TAL) as a source. Sensitivity of the Kit was determined using various concentrations of prepared endotoxin standards and pyrogen free water samples. It was observed that Endo sensor could detect up to nano gram level of endotoxin in liquid biological samples which could be expressed in (EU/ml) and the labeled sensitivity of the lysated product was 0.125 EU/ml. The gel clotting principle method was utilized for the detection of bacterial endotoxin in liquid biological samples.

Keywords: Endotoxins, *Tachypleus gigas,* Tachypleus Amebocyte Lysate (TAL), labeled sensitivity and endotoxin unit

Introduction

Gram negative bacteria are characterized by their outer membrane (cell envelop) which is chemically made up of Lipopolysaccharide (LPS). These bacteria often shed their cell wall which contains the biologically active pyrogenic substances like endotoxins (Iwanaga *et al*., 1992; John *et al*., 2010). When the host blood stream is accidentally encountered by the endotoxin, it rises the host body temperature which may be detrimental to the proper physiological functioning of the animal. In the 1920s, Rabbit Pyrogen Tests (RPT) was developed by Florence Seibert for the detection of pyrogens in any biological samples. This test involves injection of pharmaceutical parenterals drugs into a rabbit and monitoring the animal for an increase in temperature or a fever. If the rabbit spiked a fever, it indicated that the sample contained an unacceptable level of a pyrogen, such as endotoxin, and the batch of product could not be sold. In the late 1960s, researchers started studying the blood clotting mechanism of Atlantic horseshoe crab (*Limulus polyphemus*) and found that it was extremely sensitive to endotoxin. Frederick Bang and Jack Levin developed an endotoxin test that involved mixing the blood-clotting factors that are in the amebocyte with a drug sample in a test tube (Levin and Bang, 1964). If sufficient endotoxin was present, the liquid in the tube would clot in such way that when the tube was inverted top to bottom, the clot stayed in the bottom of the tube. This was the first "LAL" test for endotoxin. LAL stands for Limulus Amebocyte Lysate and contains the factors inside the amebocytes (blood cells) of the horseshoe crab, *Limulus polyphemus*. In 1977, Food and Drug Administration (FDA) approved the use of LAL test as a replacement for the Rabbit Pyrogen to detect endotoxin in human and animal injectable pharmaceuticals and biologicals, and implantable medical devices (Fink *et al.*, 1981). It is also to be noted that lysate from washed Amebocytes of horseshoe crab is 3-300 times more sensitive than rabbit pyrogen test. In recent days, Atlantic horseshoe crabs are only the sole source of these tests due to its higher population density and size compared with its conspecifics in South East Asian countries including Malaysia. *Tachypleus gigas* is one of the four extant species of horseshoe crab found in shallow water in South East Asia at depths of up to 40 m (130 ft). In Malaysia, their distribution was recorded in both the coasts including Borneo Island (Kassim *et al*., 2008; Smith *et al*., 2009a and b). The pharmaceutical application of its blood compound drove us to investigate the special type of cells in its blood (Amebocyte cells) to determine / quantify the bacterial endotoxin in liquid biologicals using Tachypleus Amebocyte Lysate (TAL) as a source in gel clotting method.

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Materials and Methods

The TAL used in this study was obtained by bleeding Malaysian horseshoe crabs (*Tachypleus gigas*) collected from Pahang coast (Figure 1). Armstrong and Conrad (2008) method was adopted to prepare TAL solution with slight modification. Labeled Lysate Sensitivity of the product was determined by double dilution method. End point dilution technique was adopted to quantify bacterial endotoxin concentration in unknown samples.

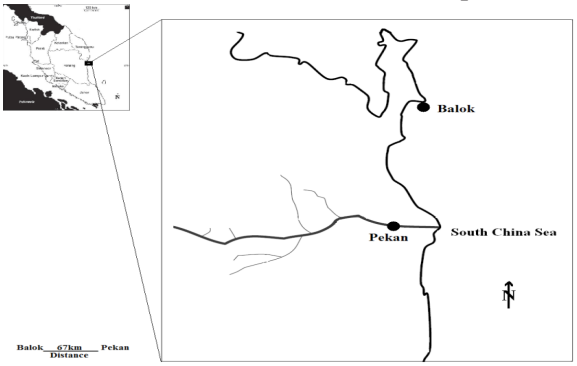

Figure 1. Location of sampling sites at Pahang coast, East coast of Peninsular Malaysia

TAL test

The TAL test was performed by mixing 0.1 ml of reconstituted TAL reagent (using endotoxin free water) and same amount of test sample in a sterile pyrogen free 10 mm × 75 mm glass test tube with an aluminum foil cover and incubated the mixture in a water bath at 37°C without any physical disturbances like shaking. The mixture were examined after 60 min; a firm gel that remained adherent to the bottom of the tube when it is inverted 180°C was interpreted as a positive test while the viscous liquid mixture after 60 min when the tube is inverted as described before was considered to be negative

Results and Discussion

The prepared Amebocyte lysate could detect as little as 1ng/ml of endotoxin in liquid biologicals which could be expressed in endotoxin unit (EU/ml). Although gel clot formation is biochemically more complex, the reaction sequence in gel clot method can be visualized as two reactions. In the presence of endotoxin proenzyme in the amebocyte lysate is converted to an active form (John *et al*., 2010). The amount of active enzyme generated is dependent upon the concentration of endotoxin originally present. The active enzyme cleaves the clotting protein, also found in the Tachypleus amebocyte. These cleaved fragments self aggregate to form clot. Typically, one incubates a tenth of a milliliter of a TAL with an equal volume of sample for 60 min at 37°C. After the incubation period, the reaction tubes were inverted. Positive results were noted when the clot remains intact after inversion of the test tube. Due to the qualitative nature of the gel clot method, to assign significance to a negative result it is essential to determine the minimum amount of endotoxin required for clot formation (John *et al*., 2010). In this study, the minimum amount of endotoxin required for clot formation was determined through double dilution of standard endotoxin and the lysate sensitivity was determined as 0.125 EU/ml. Hence test sample containing equal or more than Lysate sensitivity concentration of endotoxin could be easily determined via gel clot method. On the other hand, if the test sample contains lesser endotoxin level than the labeled lysate sensitivity, it could not be determined/ quantified using gel clot method. There are various other methods such as chromogenic substrate method (Iwanga *et al*., 1978) and kinetic turbidimetric method (Remillard *et al*., 1987) could be adopted to quantify pico gram level of endotoxin in test samples but due to their high cost in performing test, gel clot methods are quite often preferred for general use. The complete details on calculation and determination of endotoxin level in test sample is explained in Table 1, 2 and 3, where Table 1 explains the lysate sensitivity determination step using the dilution of reference standard endotoxin and the anti Log_{10} value of the mean Log value of endpoints were used as a source for lysate sensitivity determination. Table 2 gives details on test sample double dilution as per the Maximum valid dilution determined using following formula;

$$MVD = \frac{\text{Maximum Endotoxin Concentration}}{\text{Lysate sensitivity}}$$

Endotoxin concentration in liquid biologicals was determined by performing standardization step through Log and Antilog value calculation using table 2 endpoint dilution data (Table 3). The concentration of endotoxin in unknown sample was determined using the following formula: Concentration of Endotoxin = Lysate sensitivity × Endpoint dilution.

Table 1. Assay results – Gel clot method Endpoint dilution (EU/ml). Labeled lysate sensitivity = 0.125 EU/ml and Lysate sensitivity = 0.1 EU/ml

Replicate	0.5	0.25	0.125	0.06	0.03	H_20	Endpoint	Log_{10} Endpoint
1	+	+	+	-	-	-	0.125	-0.093
2	+	+	+	+	-	-	0.06	-1.222
3	+	+	+	-	-	-	0.125	-0.093
4	+	+	+	-	-	-	0.125	-0.093
							Mean	-0.983
							Anti Log_{10} mean	0.1 EU/ml

Table 2. Determination of Endotoxin concentration in an unknown sample. Double standard serial dilution of test sample as per maximum valid dilution calculation

Replicate	1/2	1/4	1/8	1/16	1/32	1/64
1	+	+	+	+	-	-
2	+	+	+	-	-	-
3	+	+	+	-	-	-

Table 3. Determination of endotoxin concentration in unknown sample

Endpoint Dilution	Log_{10} Endpoint
1/16 = 0.0625	-1.204
1/8 = 0.125	-0.903
1/8 = 0.125	-0.903
Mean	-1.003
Anti Log_{10} mean	0.099
Concentration of Endotoxin = Lysate sensitivity × Endpoint dilution = 0.125 × 10.1 = 1.26 EU/ml	

Conclusion

In Malaysia, horseshoe crab landing is observed in both east and west coasts but the scientific exploration on this animal is still scanty. We propose that Malaysian horseshoe crabs *Tachypleus gigas* could be substantially used to derive Tachypleus amebocyte lysate (TAL) from its blood which could be used to validate liquid biologicals including parenteral drugs for marketable purposes.

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