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# An Improved *In Vitro* Pyrogen Test To Detect The Presence of Endotoxin Containing Bacteria Using *Limulus Amoebocyte Lysate* Assay From Pharmaceutical Raw Product

Tanvir Bashar, Khondokar Mahzebin Shurovi , Sanjida Dilshad
Department of Microbiology, Stamford University Bangladesh

**Corresponding Author**
Khondokar Mahzebin Shurovi
Department of Microbiology,
Stamford University Bangladesh
Tel: 01720514155
E-mail: mahzebin@yahoo.com

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## ABSTRACT

Most of the environmental organism possess endotoxin which presence indicates the organisms are much lethal and for the purposes bacteriological quality of pharmaceutical raw products is much important .In these current study, 2 raw materials out of 10 were found to containing pathogenic bacteria *Escherichia coli* and *Pseudomonas* species in the culture medium, that indicates the raw materials were contaminated with the deadly organism. These two raw materials checked for the presence of Endotoxin and both provided positive gel clot by Limulas amoebocyte lysate (LAL) assay .Quality maintenance and assurance is the essential need of Drug preparation in pharmaceutical sector .The result ensure that Pharmaceutical industry should need to follow GMP and HACCP to minimize the contamination for improving the biological safety of the product in a cost-effective manner.

**Key Word:** Limulas amoebocyte lysate (LAL) assay, Gram negative bacteria, endotoxin .

## INTRODUCTION

Endotoxins are part of the outer membrane of the cell wall of Gram-negative bacteria and invariably associated with Gram-negative bacteria whether the organisms are pathogens or not. Although the term "endotoxin" is occasionally used to refer to any cell-associated bacterial toxin, it is properly reserved to refer to the lipopolysaccharide complex associated with the outer membrane of Gram-negative bacteria such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria*, *Haemophilus*, and other leading pathogens(Andersen et al .,1987). The biological activity of endotoxin is associated with the lipopolysaccharide (LPS) and toxicity is associated with the lipid component (Lipid A) and immunogenicity is associated with the polysaccharide components. The cell wall antigens (O antigens) of Gram-negative bacteria are components of LPS. LPS elicits a variety of inflammatory responses in an animal (Froon et al .,1995). Because it activates complement by the alternative (properdin) pathway, it is often part of the pathology of Gram-negative bacterial infections. However, pyrogenic substances can be produced by some gram positive bacteria, mycobacteria, fungi and also viruses, but the pyrogens produced by gram negative bacteria, i.e., the endotoxins, are of significance to the pharmaceutical industry because LPS plays an important role as a surface structure in the interaction of the pathogen with its host. (Kliegman et al., 1993).In pharmaceutical industry, Raw materials is the source of drug substances in which process water is generally use in the synthesis process. So production of biologically drug substances, incomplete removal of the microorganism during purification can result in the drug substance having high endotoxin levels (Hansbrough et al., 1993) Hence the present study was conducted in a reputed pharmaceuticals with an objective to detect the Endotoxin containing bacteria from some major raw materials that are used in Drug production.

## MATERIALS AND METHODS

### *Test sample*

Raw materials that(x) collected for routine microbiological assay in the Microbiology lab. This assay was performed on 8 raw materials for 14 days.

***Culture media used***

MacConkey broth and Cetrimide broth were used as enrichment broth. MacConkey agar was used as selective medium for the detection of coliform bacteria and Mannitol Salt agar and Cetrimide agar were used as selective medium for *Staphylococcus aureus* and *Pseudomonas* sp. For the confirmation of *E.coli*, Eosin methylene Blue (EMB) agar was used.

***Sampling procedure and Identification scheme***

Ten gram of raw material samples were weighted and transferred to a sterile beaker containing ninety (90) ml sterile distilled water to make $10^{-1}$ dilution and shake well with vortex mixer. Then 1 ml of the sample were transferred to an enrichment broth and incubated at $37^0$ C and later days streaked on Petri dish containing selective agar media .On the same time 1 ml from enrichment broth were took into blank Petri plate and then selective media poured and incubated at $37^0$ C for 18 hrs .

***Endotoxin assay***

All experimental samples were serial diluted with pyrogen free water and analyzed as follows:

1) **Conventional LAL (tube) method**: 0.1ml lysate mixed with 0.1 ml sample in a transparent disposable 10 × 75-mm glass tube and incubated at 37℃ in a water bath for 1 hr ; and

2) **Microplate LAL (plate) method**: 0.05 ml lysate mixed with 0.05 ml sample in a disposable micro titer plate and incubated at $35^0$C in a forced air incubator for 1 h.
A positive LAL test was recorded when the conventional glass tube or microwells were inverted 180 ° and the clot remained intact. To improve LAL sensitivity, doubling dilutions were made on the highest 10-fold dilution yielding a positive LAL test, and the LAL analysis was repeated (Twohy,C 1983). Positive controls (minimum *Escherichia coli* LPS concentration in pyrogen-free water for positive LAL result).

**RESULTS AND DISCUSSION**

With the development of healthcare infrastructure and increase of health awareness, Pharmaceutical sector has become one of the top priorities in today's production world. In Bangladesh maintenance of appropriate quality of pharmaceutical products is considered vital for achieving success in the global trade. But one of the major problems in the pharmaceutical industries, especially during long production runs, is keeping contaminant microorganisms out of the production area. Contaminant bacteria always multiply fast and rapidly turn a clean production run into a nasty mess. Moreover, when rogue bacteria go on a rampage, high cleanup cost, disrupted production schedule and most importantly-list revenue is faced. For this, microbiological concerns in pharmaceutical product manufacture continue to challenge the minds of those associated with their production. Pharmaceutical products are used in a variety of ways in the prevention, treatment and diagnosis of disease. It has been known for many years that microbial contaminants may affect the spoilage of pharmaceutical products through chemical, physical or aesthetic changes in the nature of the product, thereby rendering it unfit for use.

Raw material which is the key of any pharmaceutical product is needed to remain in safe and bacteria free before long scale production. Endotoxin is complex polysaccharide molecule that elicits an antigenic response, resulting in fever and altered resistance to bacterial infections .Exposure may cause toxic hemorrhagic shock and severe diarrhea. For the purposes this study was conducted on a pharmaceuticals of their some major raw materials and the result that was found is not satisfactory. Two raw materials out of 8 were found to exceed the Limit(>100 cfu) shown in Table 1 and these raw materials RM-5 (Ethacridine Lactate Specs) and RM-6(Cyclodextrin) were repeated three times and found the same result.

On the MaConkey agar, coliform bacteria (pink in color and small in size) was found in large number and their presence indicates that raw materials was highly contaminated with pathogenic bacteria .*E.coli* was identified in the EMB agar by their green metallic sheen in color Similarly green colony was also found in cetrimide agar and this indicates the presence of gram negative *Pseudomonas* spp that presented in Table 1.

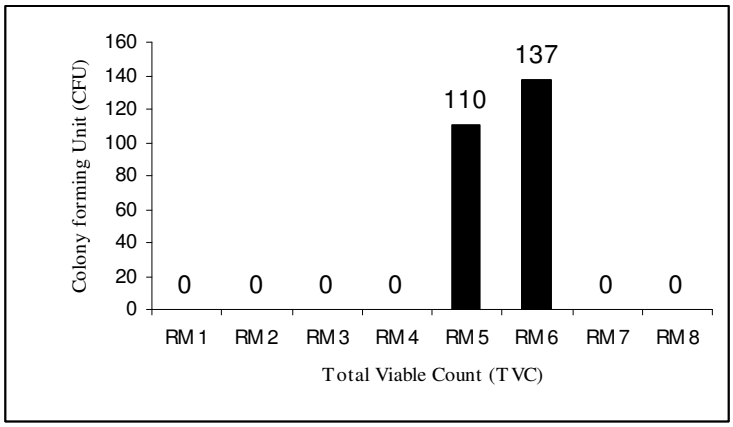


**Figure 1: Graphical presentation of total viable count of Raw materials**

**Table 1: Microbiological assay of Raw materials**

| Source | **Presence of coliform bacteria on MacConey agar** | **Presence of *Pseudomonas on*** Cetrimide agar | **Coliform bacteria on EMB agar** | **Confirmatory organism** |
|---|---|---|---|---|
| RM-1 | - ve | - ve | Not done | Not done |
| RM-2 | - ve | - ve | Not done | Not done |
| RM-3 | - ve | - ve | Not done | Not done |
| RM-4 | - ve | - ve | Not done | Not done |
| **RM-5** | **+ ve** | **+ ve** | **Green metallic sheen** | ***E.coli*** |
| **RM-6** | **+ ve** | **+ ve** | **Green metallic sheen** | ***E.coli*** |
| RM-7 | - | - | Not done | Not done |
| RM-8 | - | - | Not done | Not done |

The when the samples was performed for LAL assay only these two raw materials were found to become positive gel clot in both process, which indicates the presence of endotoxin and these endotoxin containing bacteria were found none other than *E.coli* that shown in Table 2 .

**Table 2:** Limulas amoebocyte lysate assay of Raw materials

| **Source** | **Observation of Gel clot** | | **Interpretation** |
|---|---|---|---|
| | Conventional Method | Microplate method | |
| **RM-5** | **+ ve** | **+ ve** | **Pyrogenic** |
| **RM-6** | **+ ve** | **+ ve** | **Pyrogenic** |

## CONCLUSION

It can be concluded that before production of any drugs, raw materials should need to be bacteriological analyzed because many life saving drugs administrated parentally. Presence of pyrogenic bacteria should be limited in manufacturing drugs because the safety and potency of commercial available medicines and vaccines must be guaranteed. Pharmaceutical industry should need to follow GMP and HACCP to minimize the contamination for improving the microbiological safety of the product in a cost-effective manner and Quality maintenance and assurance is the essential need of Drug preparation in this sector.

**REFERENCES**

Andersen L, Baek L, Degn H, Lehd J, Krasnik M, Rasmussen J. (1987) Presence of circulating endotoxins during cardiac operations. *J Thorac Cardiovasc Surg.* 93:115-119.

Froon A, Dentencer M, Greve JW, Raqmsay G, Buurman W. (1995) Lipopolysaccharide toxicity-regulating proteins in bacteremia. *J Infect Dis* .171:1250-7.

Hansbrough J, Tenenhaus M, Wikstrom T, Braide M, Hoffman W, Natanson C. (1993) Endotoxin in septic shock. *J Infect Dis.* 77:613-24.

Kliegman RM, Walker WA, Yolken RH. (1993) Necrotizing enterocolitis: Research agenda for a disease unknown etiology and pathogenesis.*Pediatric Research*. 34 (6): 701-708.

Twohy C. (1983) Comparison of Limulus Amebocyte Lysates from Different Manufacturers, *J Parent Sci Technol*. 37 (3): 93-94.