# DETECTION OF BACTERIAL ENDOTOXIN USING LIMULUS AMOEBOCYTE LYSATE (LAL) ASSAY FOR WATER AMPOULES AND CEFTRIAXONE SODIUM IN INDUSTRY SETUP

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**ABSTRACT**

**Background**

Endotoxin, present in the outer membrane of all Gram-negative bacteria, such as *E. coli, Salmonella, Pseudomonas, Haemophilus,* and other pathogens, causes fever, pain, irreversible shock, and even death. The Limulus amebocyte lysate (LAL) test is an alternative method to the rabbit pyrogen test focused on the detection of pyrogenic substances in sterile parenteral drugs.

**Purpose of the study**

The study aimed to detect the bacterial endotoxin using LAL tests in ceftriaxone and water for injection in an industrial setup.

**Methodology**

This is a cross-sectional study carried out at Saydon Pharmaceutical, an industrial area Peshawar from March to July 2023. Limulus Amoebocyte Lysate (gel clot method) test was performed to detect bacterial endotoxin in a total of 60 samples of water ampoules and ceftriaxone sodium in the pharmaceutical industry.

**Results**

The 60 samples of water ampoules and ceftriaxone sodium from various pharmaceuticals were analyzed to check for bacterial endotoxins. All the samples of water ampoules and ceftriaxone sodium were found to be negative for endotoxin.

**Conclusion**

In the present study, we conclude that there are no endotoxins present in the study samples, and we recommend that these injections and the water for injections are safe for human health.

**Keywords**: Endotoxin, Pyrogen, LAL, Rabbit pyrogen test

## Introduction

An endotoxin (chemical name lipopolysaccharide or LPS) is a component of the cell wall of all Gram-negative bacteria i.e. *E. coli, Salmonella, Pseudomonas, Haemophilus* (Madsen, 2001). It can activate the immune cells at very low (picogram) concentrations (Vogel *et al.*, 2005; Perkins *et al.*, 2016). The proinflammatory mediators (cytokines, leukotriene, eicosanoids, etc.) produced by the cells in response to an endotoxin are responsible for fever, hypotension, hypertension (Dobrovolskaia and Vogel, 2002; Vogel *et al.*, 2005; Perkins *et al.*, 2016), and more severe health problems including sepsis, disseminated intravascular coagulation (DIC), endotoxin shock, adult respiratory distress syndrome (ARDS), lungs function impairment, as well as the multiple organ failure (Lynn and Golenbock, 1992; Lemke, 1994; Khalid and Tahir, 2016). The severity of the immune-mediated side-effects triggered by the endotoxin depends on its potency determined by the endotoxin composition and structure and measured in international endotoxin units (Dobrovolskaia and Vogel, 2002). Endotoxin contains the main three parts: core-polysaccharide, O-antigen which causes

immunogenicity and lipid A causes toxicity (Bertics, Gavala and Denlinger, 2006; Garcia-Vello *et al.*, 2022).

On exposure to endotoxin, coagulation is formed in the amoebocyte of the horseshoe crab to restrict the attacking microorganisms, preventing following core values (Levin, 1964; Shuster Barlow and Brockmann, 2003). This clotting mechanism is a cascade containing the activation of three serine protease zymogens; factor C, factor B, and a pro-clotting enzyme and a gel forming protein (Iwanaga, 2007). To start the chain response, LPS should initially bind to factor C, permitting its enactment. The activated factor C then activates the inactive precursor of factor B to its active complement that then converts the pro-clotting enzyme to the acting clotting enzyme (Iwanaga, 1993). Coagulogen, on cleavage by the clotting enzyme, is converted to coagulin and polymerizes into a gel through noncovalent head-to-tail connections (Iwanaga, Kawabata and Muta, 1998).

The LAL tests are available in three different forms; turbidimetric, chromogenic, and gel clot techniques (Jin *et al.*, 2018). The LAL gel-clot assay is the most basic type of LAL assay. A gel equal to the endotoxin sensitivity of the given test will appear when the LAL assay is used together with a dilution of an endotoxin-containing sample. Until a negative reaction (no visible clot) is obtained, the endotoxin concentration is estimated by using a less sensitive assay. This process can take many hours (Hochstein and Seligmann 1972). However, the other two methods the chromogenic LAL method and the turbidimetric LAL method are kinetic, suggesting that these can assess the endotoxin concentration by obtaining the LAL assay's real-time responses (Novitsky, 1984).

Regular antimicrobials and their semi-synthetic aides contain the majority of anti-infection agents in clinical use; the β-lactams the first class of derivatives of natural antibiotics used in the therapeutic treatment of bacterial infections (Guimaraes, et al., 2010). Beginning around 1970, cephalosporin are among the most powerful and generally used anti-infectious agents (Fernandes et al., 2013). They β-lactam anti-infection agents have a broad spectrum of antibacterial activity, clinical viability and magnificent safety profile, following up on the chemical transpeptidase, which is unusual in bacteria which gives the activity in hindrance of bacterial wall (Guimaraes et al., 2010). Ceftriaxone sodium a third-generation semi-synthetic cephalosporin, derived from a fermentation product, for injectable use, are able to overcome the blood-brain barrier, since previous generations do not have this capacity (Amin and Ragab, 2004; Guimaraese et al., 2010; Manfio *et al.*, 2013).

Every product being injected intravenously into a human body must undergo endotoxin testing. Endotoxin testing is required for all pharmaceuticals projected for injection and all medical devices anticipated for implantation. In Pakistan a previous observation about endotoxin testing by LAL assay has been done on different samples such as airborne dust samples in Faisalabad (Khan et al., 2015). Limited published data available regarding the presence of endotoxin in water for injection as well the injectable antibiotics, which need to be, investigated because of their safety concerns. So in the present study our aim was to investigate the presence of endotoxin presence in different samples of ceftriaxone injections and water for ampules obtained from multiple pharmaceuticals in Pakistan and to ensure their sterility in order to check the safety for humans in case of injectable and antibiotic powder.

## Materials and methods

The study was carried out in Saydon Pharmaceutical, industrial area Peshawar from March to July 2023. A total of 60 samples were screened for endotoxin production. Thirty samples of ceftriaxone sodium and water for injections each, manufactured in various pharmaceutical industries in Peshawar, Islamabad, Karachi and Lahore (mentioned in Table. 1 and Table. 2 respectively) were purchased from various pharmacies in Peshawar city. The product name, manufacturer name, dose, batch number, manufacture date, expiry date and results of tests for ceftriaxone sodium, and product name, manufacturer name, volume, batch number, manufacture date, expiry date and results for water ampules were given in Table 1 and Table 2 respectively. The lyophilized kit of LAL reagent "BIOENDO" was purchased from Xiamen Bioendo Technology Co., Ltd, China.

### Preparation of Samples:

Preparation of samples was performed aseptically using depyrogenated apparatus and non-pyrogenic reagents as recommended in United State Pharmacopeia. Samples were diluted according to United State Pharmacopeia to screen for detection of bacterial endotoxin. The maximum valid dilution was calculated using the following formula previously prescribed by (Convention, 2011).

MVD = (endotoxin limit X concentration of product)/sensitivity.

### Sensitivity of Lysate:

The sensitivity of lysate was 0.25 EU/ml was labeled on Kit.

**Methodology**:

For the detection of bacterial endotoxin in water and antibiotic samples the LAL assay was used.

**Gel-clot Method:**

In this study endotoxin detection was performed using standard gel clot LAL method (Cooper et al., 1972) using a lyapholized kit of LAL reagent "BIOENDO" purchased from Xiamen Bioendo Technology Co., Ltd using the standard protocol. We performed the assay with different dilutions i.e. 200 µL for 250 mg ceftrizone sodium, 500, 800 µL for 500 mg ceftrizone sodium and 800µL for 1000 mg ceftrizone sodium and water for ampules separately.

**RESULTS**

Out of 60 samples (30 samples of ceftriaxone sodium and 30 samples of water for ampules) analyzed in various pharmaceutical industries the water for injections as well as the ceftriaxone sodium injections were found to be negative for endotoxin. All the results are collectively shown in Table. 1. And Table 2, for ceftriaxone sodium and water for injections, respectively. While table 3, indicates the result of control the standard endotoxin. 1$^{st}$, 2$^{nd}$, 3$^{rd}$, 4$^{th}$, and 5$^{th}$ dilution will give a positive result, because of its sensitivity, i.e. 25 EU/ml, 5 EU/ml, 1 EU/ml, 0.5 EU/ml and 0.25 EU/ml which is equal to and above the sensitivity of Lysate (0.25EU). The 6$^{th}$ Dilution will give negative result because its sensitivity is below the sensitivity of Lysate (0.25 EU/ml)

**Table 1. Ceftriaxone Sodium**

| S. No | Product Name | Manufacturer Name | Dose | Batch No. | Manufacturing Date | Expiry Date | Results |
|---|---|---|---|---|---|---|---|
| 1 | Geo | Saydon Pharmaceutical Industry | 250 mg | OI-623 | Sep-2020 | Sep-2023 | Negative |
| 2 | Geo | Saydon Pharmaceutical Industry | 500 mg | 2D-389 | Apr-2022 | Apr-2025 | Negative |
| 3 | Geo | Saydon Pharmaceutical Industry | 1 g | 2C-330 | Mar-2022 | Mar-2025 | Negative |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 4 | Geo | Saydon Pharmaceutical Industry | 250 mg | 1L-231 | Dec-2021 | Dec-2024 | Negative |
| 5 | Geo | Saydon Pharmaceutical Industry | 500 mg | 0I-622 | Sep-2020 | Sep-2023 | Negative |
| 6 | Geo | Saydon Pharmaceutical Industry | 1 g | 2J-525 | Oct-2022 | Oct-2025 | Negative |
| 7 | Ceftro | Highnoon Laboratories Limited | 250 mg | 2280008 | Jan-2022 | Dec-2023 | Negative |
| 8 | Ceftro | Highnoon Laboratories Limited | 500 mg | 2280070 | Jan-2022 | Dec-2023 | Negative |
| 9 | Ceftro | Highnoon Laboratories Limited | 1 g | 2280176 | Aug-2022 | Jul-2025 | Negative |
| 10 | Vexa | Neutro Pharma (Pvt) Ltd. | 250 mg | 2173P079 | Dec-2021 | Aug-2023 | Negative |
| 11 | Vexa | Neutro Pharma (Pvt) Ltd. | 500 mg | 3612P080 | Sep-2022 | Jul-2024 | Negative |
| 12 | Vexa | Neutro Pharma (Pvt) Ltd. | 1 g | 3918P078 | Dec-2022 | Nov-2024 | Negative |
| 13 | Inocef | Barrett Hodgson Pakistan (PVT) Ltd. | 250 mg | D0048 | Feb-2022 | Jan-2025 | Negative |
| 14 | Inocef | Barrett Hodgson Pakistan (PVT) Ltd. | 500 mg | D2842 | Oct-2022 | Sep-2025 | Negative |
| 15 | Inocef | Barrett Hodgson Pakistan (PVT) Ltd. | 1 g | C8727 | Oct-2021 | Sep-2024 | Negative |
| 16 | Titan | Macter International Limited | 250 mg | 1011 | Dec-2021 | Nov-23 | Negative |
| 17 | Titan | Macter International Limited | 500 mg | 22025 | Sep-2022 | Aug-24 | Negative |
| 18 | Titan | Macter International | 1 g | 22059 | Oct-2022 | Sep-24 | Negative |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | Limited | | | | | |
| 19 | Norbac | Global Pharmaceutical (Pvt) Ltd. | 250 mg | 22F122 | Jun-2022 | May-2024 | Negative |
| 20 | Norbac | Global Pharmaceutical (Pvt) Ltd. | 500 mg | 22J059 | May-2022 | Jun-2024 | Negative |
| 21 | Norbac | Global Pharmaceutical (Pvt) Ltd. | 1 g | 22K208 | Oct-22 | Mar-2025 | Negative |
| 22 | Cefxone | Bosch Pharmaceuticals (Pvt) Ltd. | 250 mg | A220367 | Oct-21 | Sep-24 | Negative |
| 23 | Cefxone | Bosch Pharmaceuticals (Pvt) Ltd. | 500 mg | A221283 | Jun-22 | May-25 | Negative |
| 24 | Cefxone | Bosch Pharmaceuticals (Pvt) Ltd. | 1 g | A230220 | Sep-22 | Aug-25 | Negative |
| 25 | Oxidil | Sami Pharmaceuticals (Pvt) Ltd. | 250 mg | 004H | Mar-2022 | Feb-2024 | Negative |
| 26 | Oxidil | Sami Pharmaceuticals (Pvt) Ltd. | 500 mg | 089H | Aug-2022 | Jul-2024 | Negative |
| 27 | Oxidil | Sami Pharmaceuticals (Pvt) Ltd. | 1 g | 089H | Aug-2022 | Jul-2024 | Negative |
| 28 | Watcef | Iqra Pharmaceuticals | 250mg | 21C107 | 03-2022 | 10-2023 | Negative |
| 29 | Watcef | Iqra Pharmaceuticals | 500mg | 21C107 | 03-2022 | 03-2023 | Negative |
| 30 | Watcef | Iqra Pharmaceuticals | 1 g | 21A002 | 01-2022 | 06-2023 | Negative |

**Table 2. Water for Injection**

| S. No | Product Name | Manufacturer Name | Volume | Batch No. | Manufacturing Date | Expiry Date | Results |
|---|---|---|---|---|---|---|---|
| 31 | WFI | Barrett Hodgson Pakistan (PVT) Ltd. | 10 ml | C8474 | Sep-2021 | Sep-2026 | Negative |
| 32 | WFI | Macter International Limited | 10 ml | 22037 | Sep-2022 | Aug-2025 | Negative |
| 33 | WFI | FYNK Pharmaceuticals | 5 ml | WI-417 | Oct-2019 | Oct-2022 | Negative |
| 34 | WFI | Neutro Pharma (Pvt) Ltd. | 5 ml | 8611W22 | Nov-2022 | Nov-2024 | Negative |
| 35 | WFI | Vision Pharmaceuticals Manufacturing Unit | 5 ml | J-22429 | Sep-2022 | Sep-2025 | Negative |
| 36 | WFI | Saydon Pharmaceutical Industries (Pvt) Ltd. | 5 ml | 2H-439 | Sep-2022 | Sep-2027 | Negative |
| 37 | WFI | Saydon Pharmaceutical Industries (Pvt) Ltd. | 5 ml | 2H-439 | Sep-2022 | Sep-2027 | Negative |
| 38 | WFI | Saydon Pharmaceutical Industries (Pvt) Ltd. | 5 ml | 2H-439 | Sep-2022 | Sep-2027 | Negative |
| 39 | WFI | Saydon Pharmaceutical Industries (Pvt) Ltd. | 5 ml | 2H-439 | Sep-2022 | Sep-2027 | Negative |
| 40 | WFI | Saydon Pharmaceutical Industries (Pvt) Ltd. | 5 ml | 2H-439 | Sep-2022 | Sep-2027 | Negative |
| 41 | WFI | Saydon Pharmaceutical Industries (Pvt) Ltd. | 5 ml | 2H-439 | Sep-2022 | Sep-2027 | Negative |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 42 | WFI | Saydon Pharmaceutical Industries (Pvt) Ltd. | 5 ml | 2H-439 | Sep-2022 | Sep-2027 | Negative |
| 43 | WFI | Saydon Pharmaceutical Industries (Pvt) Ltd. | 5 ml | 2H-439 | Sep-2022 | Sep-2027 | Negative |
| 44 | WFI | Saydon Pharmaceutical Industries (Pvt) Ltd. | 5 ml | 2H-439 | Sep-2022 | Sep-2027 | Negative |
| 45 | WFI | Saydon Pharmaceutical Industries (Pvt) Ltd. | 5 ml | 2H-439 | Sep-2022 | Sep-2027 | Negative |
| 46 | WFI | Saydon Pharmaceutical Industries (Pvt) Ltd. | 5 ml | 2H-439 | Sep-2022 | Sep-2027 | Negative |
| 47 | WFI | Sami Pharmaceuticals (Pvt) Ltd. | 5ml | 087H | Mar-22 | Feb-25 | Negative |
| 48 | WFI | Sami Pharmaceuticals (Pvt) Ltd. | 5ml | 249H | Aug-22 | Jul-25 | Negative |
| 49 | WFI | Surge Laboratories (Pvt) Ltd. | 10 ml | WF2-699B | Oct-20 | Oct-25 | Negative |
| 50 | WFI | Iqra Pharmaceuticals | 5ml | 20F126 | 06-2021 | 06-2023 | Negative |
| 51 | WFI | Bosch Pharmaceuticals (Pvt) Ltd. | 5 ml | WI-220283 | Apr-22 | Mar-26 | Negative |
| 52 | WFI | Sanofi Pharmaceutical | 5ml | AW050 | Apr-2021 | Mar-2026 | Negative |
| 53 | WFI | P.D.H Laboratories Pvt Ltd. | 5ml | PE220303 | Mar-2022 | Feb-2024 | Negative |
| 54 | WFI | Gray's Pharmaceuticals | 5ml | 072457 | Apr-2021 | Mar-2023 | Negative |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 55 | WFI | Zafa Pharmaceuticals Pvt Ltd. | 5ml | 044 | Aug-2020 | Aug-2025 | Negative |
| 56 | WFI | Asian Continental | 5ml | I-368C | Jun-2021 | Jun-2026 | Negative |
| 57 | WFI | Global Pharmaceutical Pvt Ltd. | 5ml | 21M040 | Dec-2021 | Nov-2024 | Negative |
| 58 | WFI | Surge Pharma | 5ml | SF3-871B | 10-2020 | 04-2023 | Negative |
| 59 | WFI | Highnoon Pharma | 5ml | W-1706 | Aug-2021 | Jul-2024 | Negative |
| 60 | WFI | Bosch Pharmaceutical Pvt Ltd. | 5ml | w-1220079 | 09-2021 | 08-2025 | Negative |

**Table. 3. Results of Control Standard Endotoxin**

| S. No. | Control Standard Endotoxin | Endotoxin Sensitivity | Clot Formation | Result |
|---|---|---|---|---|
| 1 | 1st Dilution | 25 EU/ml | YES | + |
| 2 | 2nd Dilution | 5 EU/ml | YES | + |
| 3 | 3rd Dilution | 1 EU/ml | YES | + |
| 4 | 4th Dilution | 0.5 EU/ml | YES | + |
| 5 | 5th Dilution | 0.25 EU/ml | YES | + |
| 6 | 6th Dilution | 0.125 EU/ml | NO | - |

## Discussion:

In the current study, 60 samples of water for injections and ceftriaxone sodium injections of different pharmaceutical industries situated in Peshawar, Karachi, Islamabad, and Lahore were screened for detection of bacterial endotoxin; all the samples were found to be negative. In a similar study from Hyderabad, India (Chaitanya et al., 2013) performed a LAL test for detection of bacterial endotoxins like *E. coli*, *Salmonella*, *Pseudomonas*, and *Hemophilus*, the various samples of products like the pharmaceutical compounds cloxacillin sodium sterile, sulbactam sodium sterile, ceftriaxone sodium sterile, piperacillin sodium sterile, and amoxicillin sodium sterile are tested for the endotoxins and have been observed to be negative for endotoxin presence. In another study, reported from Charleston, United States by (Dubczak et al., 2021), a total of 128 samples of water for injection were screened for

detection of bacterial endotoxin using various tests including, the LAL test, Charles River, LAL Lonza, LAL Pyrogen, they found that all the results were negative. Similarly, other reports supported the current findings from Hangzhou City, China (He et al., 2012), and the tachypleus amoebocyte lysate (gel-clot method) was used to detect the bacterial endotoxin in Rabies Vaccine (KMB17 cell) for human use; all the results were negative.

A contrasting report from Pakistan suggested that out of twenty-five high volume parenterals, such as distilled water, metronidazole infusions, mannitol, electrolytes, dextrose, and water for injections, one metronidazole injection out of several preparations showed a positive LAL test; and furthermore, that injection was determined to be pyrogen free using the rabbit pyrogen test (Naqvi *et al.*, 2004). A recent study from Nigeria by (Ezugwu et al., 2023), in which 120 brand samples of pentazocine and diazepam (60 each) were screened for bacterial endotoxin test and found that the 7 samples (5 samples of pentazocine and 2 samples of diazepam) was positive for bacterial endotoxin using Gel-Clot method. A dissimilar study from Basel, Switzerland by (Arnet et al., 2015), the bacterial endotoxins were determined using the quantitative kinetic-chromogenic LAL method and reported that, in 8 different generics of ceftriaxone, endotoxins were present 10 EU/vial in all the generic products, and none of them exceed the EU pharmacopoeia specifications. The presence of bacterial toxin in injectable poses a major health issue that must be monitored in order to maintain safety precautions.

## Conclusion

The results obtained showed that samples of ceftriaxone sodium and water for injections used were found negative for endotoxin presence. These study findings suggested that the ceftriaxone injections and water for ampules (used in our study) are safe to use for therapeutic purposes.

## Conflict of interests

Authors and the co-authors declare that there is no conflict of interest and this submission is an original work.

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