MedCrave
Step into the World of Research
Journal of Applied Biotechnology and Bioengineering

Review Article

Open Access
CrossMark

Endotoxin testing: past and contemporary methods, ecological impact, and developing of alternatives

Abstract

In the biotechnology industry, endotoxin testing is a ubiquitous procedure that has a fascinating history. It has impacted not only patients, but also the environment, and has the potential to be improved upon in the future. It is crucial that any drugs introduced to the body maintain a high degree of sterility, lest the drug designed to save a life is the cause of a different life-threatening disease. The predominant method used today is Limulus Amoebocyte Lysate, commonly referred to as LAL, which uses horseshoe crab blood. Since LAL is obtained by harvesting the blood of live horseshoe crabs, there is an ecological impact that comes with this type of endotoxin testing. Endotoxin testing with LAL is a vital and effective method of ensuring the safety of drug products, but it is a limited resource. Because of its necessity, it is pertinent that we develop alternatives to LAL in case it is no longer a viable option. Any alternatives should have an efficacy similar to that of LAL, but should not depend as heavily on harvesting the blood of horseshoe crabs since it is unsustainable in the long term.

Keywords: limulus amoebocyte lysate, endotoxin, horseshoe crab, pyrogen, rabbit

Volume 11 Issue 1 - 2024

Tian Jianning, Tawil Bill
Department of Biology, California State University, USA

Correspondence: Bill Tawil, Department of Biology, Channel Islands (CSUCI), USA, Email btawi@seas.ucla.edu

Received: January 25, 2024 | **Published:** February 7, 2024

Abbreviations: LAL, limulus amoebocyte lysate; LPS, lipopolysaccharide; RPT, rabbit pyrogen test; FDA, food and drug administration; HPLC, high pressure liquid chromatography; rFC, recombinant factor C

Introduction

While humans have become the dominant multicellular organism species on this planet, the microscopic world is dominated by bacteria, viruses, and certain fungi. While viruses and fungi pose risks to biopharmaceutical drug production, the major concern is bacterial contamination, specifically lipopolysaccharide (LPS) endotoxins which are produced by gram-negative bacteria.[1] These endotoxins can be found on the outer membrane of gram-negative bacteria and consist of three parts: lipid A, a core made of oligosaccharide, and an O-specific polysaccharide.[2] The lipid A region is responsible for many biological activities but the main one of concern is that it makes the endotoxins a type of pyrogen, meaning that when introduced to a host intravenously it can result in a fever.[3] Since many drugs are given intravenously, it is important to test the final product for endotoxins to minimize the chance of inadvertently inducing a fever in patients. Since drugs are manufactured to treat certain diseases in patients, not ensuring that the final product is free of endotoxins not only goes against the goal of treating patients, but could also be detrimental or fatal to an ill patient. This makes endotoxin testing a crucial step in the drug development process.

History of endotoxin testing

Early endotoxin testing methods

While the goal of endotoxin testing has remained the same, to test solutions for pyrogenicity, the methods used early on are quite different from the ones used in the present. The earliest form of endotoxin testing can be traced to 1912 when E.C. Hort and W.J. Penfold used carefully bred rabbits to test for the onset of fevers.[4] The rabbits were injected with water intravenously and their temperatures were taken at regular intervals.[4] With this method, the live rabbits were the endotoxin testing component, from which they found that freshly distilled water did not induce fevers after being injected intravenously.[5] A decade later in 1923, Florence Seibert utilized the same rabbit breed that Hort and Penfold did to conclude that the fevers observed in the rabbits originated from bacteria.[4] Although Hort, Penfold, and Siebert were performing endotoxin testing, the first wide scale endotoxin testing did not emerge until 1942.[4] The Rabbit Pyrogen Test (RPT) again involved the breed of rabbits that Hort, Penfold, and Seibert used but this time, after the rabbits had been given intravenous injections, the rectal temperatures were taken for a 3 hours to determine if the injected solution was pyrogenic.[4] Although the RPT was dependable enough to have been used for over 40 years to test the pyrogenicity of solutions, a new discovery in the 1960s revolutionized endotoxin testing.

Limulus amoebocyte lysate test

The current and by far the most extensively used method of endotoxin testing is Limulus Amoebocyte Lysate or LAL. The Limulus part of LAL refers to the family Limulidae which are known commonly as horseshoe crabs. The amoebocyte part refers to the type of cell found in horseshoe crab blood that is necessary to conduct this assay. Finally, lysate refers to how the amoebocytes are broken apart in order to obtain the necessary molecules involved in LAL testing. In the 1960s, Jack Levin and Frederik Bang discovered that horseshoe crab blood clots when exposed to bacterial endotoxin.[4] After reporting that was cheaper and had higher sensitivity to endotoxins than RPT, Levin and Bang sparked interest in replacing RPT with LAL to test for endotoxin. In 1977, the U.S. Food and Drug Administration (FDA), approved the use of LAL to test for endotoxins in the place of RPT.[6]

Horseshoe crabs are an ancient species, with origins dating back to 440 million years ago and the modern horseshoe crab appearing in the fossil record 250 million years ago. Because of this, they are often described as “living fossils”.[7] Since they have been around for so long, they are extremely sensitive to infections since they have not evolved much in hundreds of millions of years. In addition, their circulatory system is open which means any infection can quickly travel throughout their bodies, further necessitating a sensitive immune system. The blood of horseshoe crabs is also special as it appears blue due to a copper-containing molecule in their blood known as hemocyanin, as opposed to the iron-containing hemoglobin which gives our blood its red color.

In order to obtain LAL from horseshoe crabs, they are first caught and drained of their blood. This involves removing around one-third of the blood contained in each horseshoe crab and has a mortality rate of around 30%.[8] The lysate obtained from the horseshoe crabs contains a mixture of proteins that, when exposed to bacterial endotoxin, undergo an enzymatic cascade. This is essentially a chain reaction of proteins activating each other. The end of this cascade is a gel-like substance of coagulate which is used to produce coloration. This coloration is what is detected and correlates to the amount of endotoxin in the sample.[9]

Ecological impact of LAL testing

As useful as LAL testing is, the harvesting of so many horseshoe crabs for their blood can certainly take its toll on horseshoe crabs and their ecosystems. As mentioned above, there is a significant mortality rate in horseshoe crabs in the blood draining process.[8] Since it was discovered that horseshoe crab blood is an unmatched resource to test for endotoxin, the number of horseshoe crabs harvested saw a dramatic increase. Combined with loss of habitat due to human intervention, the population of horseshoe crabs has been on the decline for the past few decades.[10] Having survived for hundreds of millions of years and some major extinction events, it is sad to see these resilient creatures meet their match in humans. The overharvesting of horseshoe crabs not only affects their population but also the population of other species in their habitats. Horseshoe crab eggs are an important food source for shorebirds and the overharvesting of horseshoe crabs has led to fewer eggs to feed shore birds, whose population has also declined significantly.[7] One estimate places the number of shorebirds in Delaware Bay as decreasing from 1.5 million in the 1980s to around 200,000 in 2022.[7] The number of harvested horseshoe crabs in this area was estimated to have increased from 100,000 per year in 1992 to 2.5 million per year in 1997.[7] In 2021, the number of horseshoe crabs harvested per year was estimated to be around 740,000 is a much more sustainable number.[10] While the decrease in number of horseshoe crabs being harvested per year gives them an opportunity to recover somewhat from the drastic decline in their population, it does not guarantee their survival in the long run.

Alternatives for the future

While it is important to be conscientious about the impact of LAL testing on horseshoe crab populations, there still needs to be robust methods for testing for endotoxins to help ensure the success of biopharmaceutical products. One of the leading contenders for alternative LAL testing methods is recombinant Factor C or rFC. Factor C is a part of the enzyme cascade involved in LAL testing and after DNA of Factor C was cloned and the protein was created synthetically, it became possible to conduct an animal-free, modified version of LAL.[11] Although this method and other recombinant technologies were promising, their efficacy compared to LAL makes them less appealing alternatives.[11]

There are also some methods of endotoxin testing that do not require the use of LAL. One of these methods is gas chromatography-mass spectrometry or GS/MS. This method involves tagging the 3-hydroxy fatty acid molecules in the lipid A part of the LPS and measuring the fluorescence produced while correlating it to an endotoxin amount.[12] However, this method requires a complicated prepping stage with high pressure liquid chromatography (HPLC) and lacks sufficient sensitivity and accuracy compared to LAL.[12] Another non-LAL method is cell-based assay. This method utilizes immune cells and cell lines like neutrophils, monocytes, and human embryonic kidney cells and their response to LPS to quantify the level of endotoxins present in a solution.[12] While promising, this method has limited/low specificity against endotoxins and thus, like GC/MS, does not measure up to the efficacy of LAL.[12] Despite these limitations, the development of LAL alternatives and even the evolution of endotoxin testing itself demonstrates that science is constantly improving, and new discoveries can always be made.[13–22]

Conclusion

Endotoxin testing is a crucial step in ensuring that patients receive drug products with as little contamination as possible. The history of endotoxin testing is relatively brief, spanning from the early 1910s to the late 1970s, but has seen significant changes since its inception. The earliest endotoxin testing methods involved intravenously injecting specially bred live rabbits with solutions and observing for any fever. While injecting rabbits is a valid method, it often took hours to produce a result and required maintaining a population of specially bred rabbits. After the discovery that horseshoe crab blood clots in the presence of endotoxin, research went into determining if it could be used as a viable alternative to using rabbits. LAL proved to be cheaper, more effective, and quicker at producing results which led to it becoming the predominant method of testing for endotoxins. Unlike injecting live rabbits and observing the development of fevers, LAL utilizes protein interactions when endotoxin is introduced and the observed color at the end of these interactions is correlated to the amount of endotoxin present.

The sensitivity of horseshoe crab blood to bacterial endotoxins makes LAL a robust endotoxin testing method, but sourcing horseshoe crab blood comes with consequences. There is a significant mortality rate among horseshoe crabs during the harvesting process because the blood needs to be drained while they are still alive. Although more consideration has been given to the number of horseshoe crabs harvested each year, their population is still lower than what it used to be. The decrease in horseshoe crab population also affects their ecosystem since shorebirds rely on their eggs for food and a decrease in horseshoe crab populations has led to a decrease in shorebird populations.

Given the decline in horseshoe crab population and that horseshoe crabs are not an unlimited resource, there are alternatives in development that aim to eliminate the need to harvest horseshoe crab blood. Some methods like rFC utilize gene cloning to create a modified version of LAL, requiring far fewer horseshoe crabs, while other methods such as GS/MS and cell-based assays do not require horseshoe crabs at all. However, in order for these methods to be viable replacements for LAL on a large scale, we need to improve their efficacy to a level comparable to LAL. Nevertheless, these methods prove that testing for endotoxin is possible without relying on harvesting horseshoe crab blood and there is hope that we can help treat diseases in our own population without affecting the populations of other species.

Acknowledgements

Jianning Tian extends his immense gratitude to Dr. Tawil for assisting in arranging the publication of this paper and for sharing a wealth of knowledge about the biotechnology industry.

Conflict of interest

Authors declare that there is no conflict of interest.

Funding sources

There is no funding to report for this study.

Citation: Jianning T, Bill T. Endotoxin testing: past and contemporary methods, ecological impact, and developing of alternatives. *J Appl Biotechnol Bioeng.* 2024;11(1):6–8. DOI: 10.15406/jabb.2024.11.00351

References

1. Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. *Annual Review of Biochemistry*. 2002;71(1):635–700.
2. Galanos C, Freudenberg MA. Bacterial endotoxins: Biological properties and mechanisms of action. *Mediators of Inflammation*. 1993a;2(7).
3. Birrer GA, Murthy SS, Liu J, et al. Parenteral dosage forms. Handbook of modern pharmaceutical analysis. 2001;269–305.
4. Dubczak J. Past, present, and future of endotoxin testing. American pharmaceutical review. 2020.
5. Hort EC, Penfold WJ. The relation of Salvarsan fever to other forms of injection fever. *Proceedings of the Royal Society of Medicine*. 1912.
6. Library of Congress.
7. Decline of an ancient mariner. National Wildlife Federation.
8. Mortality in female horseshoe crabs (*Limulus polyphemus*).
9. Iwanaga S. Biochemical principle of limulus test for detecting bacterial endotoxins. *Proceedings of the Japan Academy. Series B, Physical and biological sciences.* 2007;83(4):110-119.
10. Atlantic states marine fisheries commission. Species - Atlantic States Marine Fisheries Commission.
11. Maloney T, Phelan R, Simmons N. Saving the horseshoe crab: A synthetic alternative to horseshoe crab blood for endotoxin detection. *PLOS Biology.* 2018a;16(10).
12. Tamura H, Reich J, Nagaoka I. Outstanding contributions of LAL Technology to pharmaceutical and Medical Science: Review of methods, progress, challenges, and future perspectives in early detection and management of bacterial infections and invasive fungal diseases. *Biomedicines*. 2021;9(5):536.
13. Accurate pyrogen testing. MAT Research. 2023a.
14. Affairs O. Bacterial endotoxins/pyrogens. U.S. Food and Drug Administration.
15. Alternative endotoxin testing methods. The horseshoe crab: Natural history, anatomy, conservation and current research.
16. Cascade reactions of LAL triggered in the presence of endotoxins.
17. Endotoxin timeline. The horseshoe crab: Natural history, anatomy, conservation and current research.
18. Gorman R. Atlantic horseshoe crabs and endotoxin testing: Perspectives on alternatives, sustainable methods, and the 3Rs (replacement, reduction, and refinement). *Frontiers in Marine Science*. 2020;7.
19. The history of Limulus and endotoxin.
20. Horseshoe crab abundance. Long Island sound study. 2023.
21. Horseshoe crab blood: The miracle vaccine ingredient that's saved millions of lives. Natural History Museum.
22. Horseshoe Crab. National Wildlife Federation.

Citation: Jianning T, Bill T. Endotoxin testing: past and contemporary methods, ecological impact, and developing of alternatives. *J Appl Biotechnol Bioeng.* 2024;11(1):6–8. DOI: 10.15406/jabb.2024.11.00351